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Culture and transplantation of spermatogonial stem cells

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ABSTRACT

The spermatogonial transplantation technique was developed by Dr. Ralph Brinster in 1994. Transplanted spermatogonial stem cells (SSCs) produce germ cell colonies after microinjection into the seminiferous tubules of infertile mice. This technique provided the first functional assay for SSCs. Although it became possible to produce transgenic animals using this transplantation technique in 2001, the lack of SSC culture systems prevented efficient genetic manipulation. To overcome this problem, a long-term SSC culture technique was developed in 2003. Cultured SSCs, designated as germline stem cells, allow drug selection of transfected SSCs, and knockout mice were produced in 2006. Using these techniques, it is now possible to address basic biological questions of SSC biology. They also open up new possibilities for male germline manipulation. In this review, we will briefly summarize our findings on SSCs and discuss unresolved issues that remain to be addressed.

1. Spermatogonial transplantation and germline stem cells (GSCs)

Spermatogonial stem cells (SSCs) provide the foundation for spermatogenesis. In mice, SSCs comprise 0.02–0.03% of total germ cells in testes and are thought to be localized to a specialized microenvironment called the germline niche (Meistrich and Beek, 1993; de Rooij and Russell, 2000). SSCs are defined by their two distinct functions demonstrated in the germline niche: "production of differentiating spermatogonia toward spermatogenesis (differentiation)" and "production of SSC itself (self-renewal)".

In 1994, Brinster et al. established a spermatogonial transplantation technique that allowed functional identification of SSCs (Brinster and Zimmermann, 1994). SSCs transplanted into infertile recipient testes colonize seminiferous tubules and reinitiate spermatogenesis to generate offspring (Brinster and Avarbock, 1994) (Fig. 1). It was later shown that germ cell colonies develop from single SSCs, demonstrating that this technique is applicable to quantitate SSC numbers (Zhang et al., 2003; Kanatsu-Shinohara et al., 2006b).

Although SSCs can be infected by retroviral/lentiviral/adenoviral vectors to modify the rodent genome both *in vitro* and *in vivo* (Nagano et al., 2001; Hamra et al., 2002; Kanatsu-Shinohara et al., 2004d; Takehashi et al., 2007) (Fig. 2A), the lack of an SSC culture technique limited the extent of genetic manipulations and biochemical analyses that could be performed with SSC. However, discovery of glial cell line-derived neurotrophic factor (GDNF) in the regulation of SSC self-renewal led to the development of a long-term SSC culture technique (Meng et al., 2000; Kanatsu-Shinohara et al., 2003a). Pup testis cells cultured on mouse embryonic fibroblasts (MEFs) with cytokines including GDNF form grape-like colonies (Fig. 3A). These cells, designated as germline stem cells (GSCs), not only proliferate logarithmically for more than 2 years but also reinitiate spermatogenesis and produce offspring after transplantation (Kanatsu-Shinohara et al., 2005a). Similar cultures can also be established from adult testes (Kanatsu-Shinohara et al., 2004a; Ogawa et al., 2004; Kubota et al., 2004). Using GSC cultures, transgenic and knockout (KO) mice have been produced by *in vitro* drug selection of genetically modified GSCs (Kanatsu-Shinohara et al., 2005b, 2006a) (Fig. 2B). With the development of spermatogonial transplantation and GSC culture techniques, it is now possible to expand SSCs that can be genetically modified and clonally selected to produce offspring.

2. Cytokines regulating SSC self-renewal

It was difficult to analyze SSCs by biochemical and molecular approaches because of their small population size. However, the GSC culture system allowed us to generate a large number of SSCs for analyses. Using GSC cultures, we initially focused on GDNF to understand the SSC self-renewal mechanism. We found that GDNF activates the phosphatidylinositol 3-kinase-AKT pathway, and that overexpression of constitutively active AKT enables GSCs to proliferate under GDNF-free conditions, demonstrating that GDNF-mediated AKT pathway activation is critical for self-renewal (Lee et al., 2007; Oatley et al., 2007). Subsequently, we found that GDNF activates the RAS proto-oncogene

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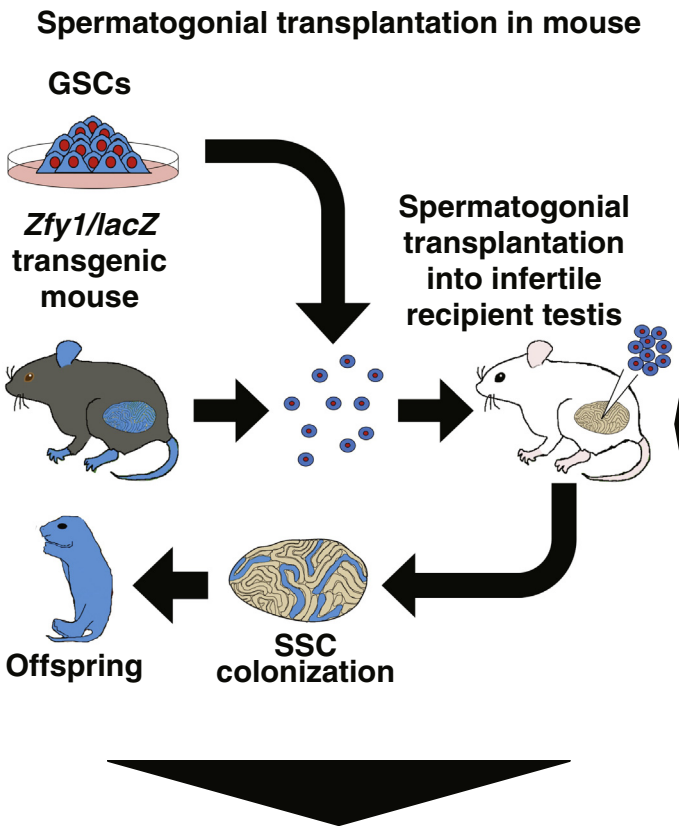

Natural mating

Brinster and Averbock., 1994
Three Bus recipients had restored fertility at least 8 months after spermatogonial transplantation

Ogawa et al., 2000
W adult recipients had restored fertility at 5 months after transplantation of SSCs from an Sl donor

Shinohara et al., 2001
W pup recipients sired offspring at 3 months after spermatogonial transplantation in all three trials, whereas W adult recipients were infertile even after SSC transplantation in all six cases

Kanatsu-Shinohara et al., 2016b
Bus recipients had restored fertility at 99 days after GSC transplantation in all seven cases

Microinsemination

Kanatsu-Shinohara et al., 2003b
Allogeneic offspring produced by SSC transplantation into histoincompatible Bus or W pup testes in combination with microinsemination

Shinohara et al., 2006
Xenogeneic offspring produced by rat SSC transplantation into busulfan-treated nude mouse testes in combination with microinsemination

Application to non-rodent species with successful production of offspring

Honaramooz et al., 2003
SSCs from transgenic goat producing human α_1-antitrypsin were transplanted into prepubertal recipient goats without both germ cell depletion and immunosuppression

Herrid et al., 2009
SSCs from donor sheep were transplanted into irradiated sheep recipient testes without immunosuppression

Lee et al., 2006
SSCs from Korean Ogol chickens with a recessive pigmentation inhibitory gene were transplanted into White Leghorn recipient carrying dominant pigmentation inhibitory gene without germ cell depletion

Trefil et al., 2006
SSCs from Black Minor chiken with black feather were transplanted into germ cell-depleted White Leghorn chicken

Fig. 1. Spermatogonial transplantation-mediated fertility restoration. In 1994, transplantation of testis cells into Bus recipients restored fertility, but its efficiency was low. Ogawa et al. demonstrated that W recipient is superior to Bus recipient for SSC transplantation from Sl donors. Moreover, it was demonstrated that W pups are superior to W adult recipients for derivation of offspring. To avoid the technical difficulties in handling pup recipients, we recently used GSCs and Bus mice, and found that offspring are born efficiently. The microinsemination technique was first used to derive offspring after allogeneic testis cell transplantation. It was also useful to derive offspring from xenogeneic rat donor cells. Although several successful cases of fertility restoration have been reported in non-rodent strains, the efficiency was very low, which requires improvement.

via a Src family kinase. Transfection of HRASV12 (constitutively active mutant) or its down-stream target *Ccnd2* in combination with *Ccne1* permit GSC proliferation and increase SSC frequency without cytokines, demonstrating that self-renewal signals act *via* the RAS-AKT-cyclin pathway (Lee et al., 2009a). Surprisingly, HRAS also appears to play a similar role in self-renewal of SSCs in humans, because activating mutations in HRAS contribute to the paternal age effect (Goriely and Wilkie, 2012).

Previous studies suggested that fibroblast growth factor (FGF) 2 contributes to SSC self-renewal (van Dissel-Emiliani et al., 1996; Kanatsu-Shinohara et al., 2003a). However, particular function of FGF2 on SSC remains to be clarified. Although it is established that GDNF and FGF2 simultaneously activate both AKT and MAP2K1 pathways in somatic cells, we found that FGF2 activates the MAP2K1 pathway more strongly than GDNF in GSCs. In addition, overexpression of the constitutively active form of MAP2K1 enables self-renewal of SSCs without FGF2. Because overexpression of downstream transcription factors ETV5 and BCL6B allows GSCs to proliferate without FGF2, it was considered that activation of the FGF2-mediated MAP2K1 pathway stimulates SSC self-renewal *via* upregulation of ETV5 and BCL6B (Oatley et al., 2006; Ishii et al., 2012).

Although reactive oxygen species (ROS) are generally detrimental to spermatogenesis, we unexpectedly found that ROS inhibitors suppressed GSC proliferation. Because GSCs overexpressing HRASV12 produce significant amounts of ROS despite their active self-renewal, we reasoned that ROS play an important role in SSC self-renewal. We

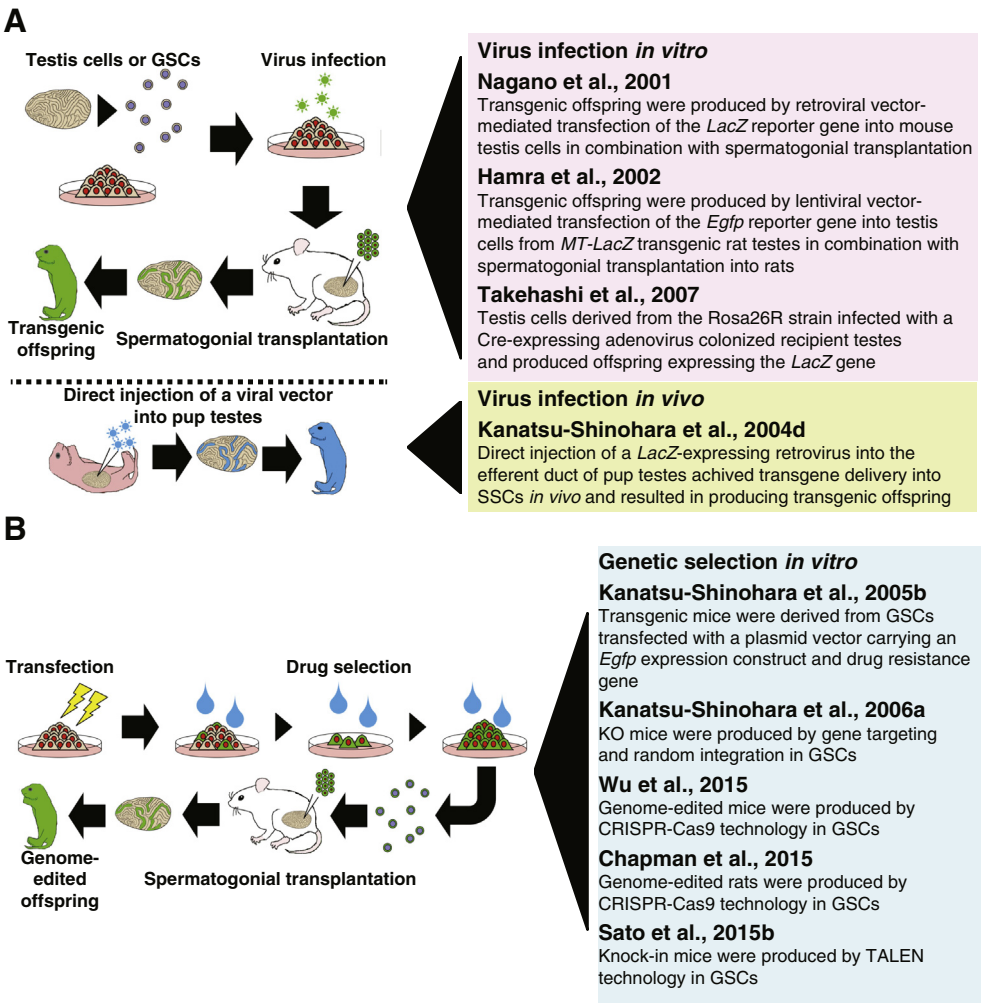

Fig. 2. Genetic manipulation of SSCs. (A) Viral vector-mediated production of transgenic offspring. The first transgenic offspring were born using retroviral transfection. A lentivirus and adenovirus were also applied. Direct injection of the retrovirus into pup testes also produced transgenic offspring. SSCs in pup testes are permissive for virus infection because of the immature BTB. (B) Production of KO animals *via* GSC culture. Development of the GSC culture technique allowed genetic selection of a transfected GSC clone. Taking advantage of this property, KO mice were eventually produced by homologous recombination in GSCs. Recently, the CRISPR-Cas9 technique has been applied to produce genome-edited mice and rats in a similar manner.

then focused on *Nox* genes and found that *Nox1* and *Nox3* are responsible for SSC self-renewal (Morimoto et al., 2013, 2015). Although excessive ROS are apparently toxic, our results suggest that self-renewal of SSCs is supported by moderate levels of ROS generated by *Nox* genes.

Although it has been taken for granted that GDNF is indispensable for SSCs (Meng et al., 2000), we recently revisited this issue because a lack of SSCs in *Gdnf* KO mice was not conclusively demonstrated by the transplantation assay in the original study. Mutant mice harboring the Y1062F null mutation in the RET proto-oncogene exhibit a Hirschsprung's disease-like phenotype including the loss of spermatogenesis, and the Y1062F mutation is thought to be critical for SSC self-renewal (Jain et al., 2004; Jijiwa et al., 2008). However, our in-depth analysis revealed survival of $GFRA1^{+}CDH1^{+}$ spermatogonia in these mutant mice. We also found that SSCs could be expanded with single growth factors GDNF or FGF2 *in vitro* (GDNF-cultured spermatogonia: G-SPG; FGF2-cultured spermatogonia: F-SPG). Although these cells formed colonies on laminin-coated dish with distinct morphologies, both of them could maintain SSC activity and restore fertility of infertile mice by spermatogonial transplantation even after 4 months of culture, demonstrating that GDNF is dispensable for self-renewal division (Takashima et al., 2015) (Fig. 3A). Interestingly, G-SPG exhibit MAP2K1 pathway-dependent self-renewal, whereas F-SPG survive and proliferate without MAP2K1 activation, indicating a functional difference between FGF2 and GDNF. So far, we have not observed significant difference in colonization efficiency after long-term culture of both cell types.

These results revealed that GDNF is not the only self-renewal factor for SSCs. There are probably additional molecules involved in SSC self-renewal, such as FGF8, CSF1, WNT3A, WNT5A, WNT6, and VEGFA (Oatley et al., 2009; Yeh et al., 2011, 2012; Lu et al., 2013; Hasegawa and Saga, 2014; Takase and Nusse, 2016; Tanaka et al., 2016). How these molecules regulate SSCs will be an important future topic. Analysis of KO mice is generally helpful, but the example using RETY1062F mutant mice suggests that caution is necessary to interpret the phenotypes of KO mice. Because Cre expression is toxic to SSCs (Kanatsu-Shinohara et al., 2008c), analysis of conditional KO mice using Cre causes additional problems, including lineage tracing studies. This is particularly true when a transplantation assay is not employed in the analysis.

3. Negative regulator of SSC self-renewal

Although much progress has been made in the characterization of positive regulators of SSC self-renewal, little is known about the negative regulators. The doubling time of GSCs is ~2.5 days *in vitro*, which is apparently shorter than that of undifferentiated spermatogonia *in vivo*. Therefore, it is not surprising that SSC self-renewal is negatively regulated *in vivo*.

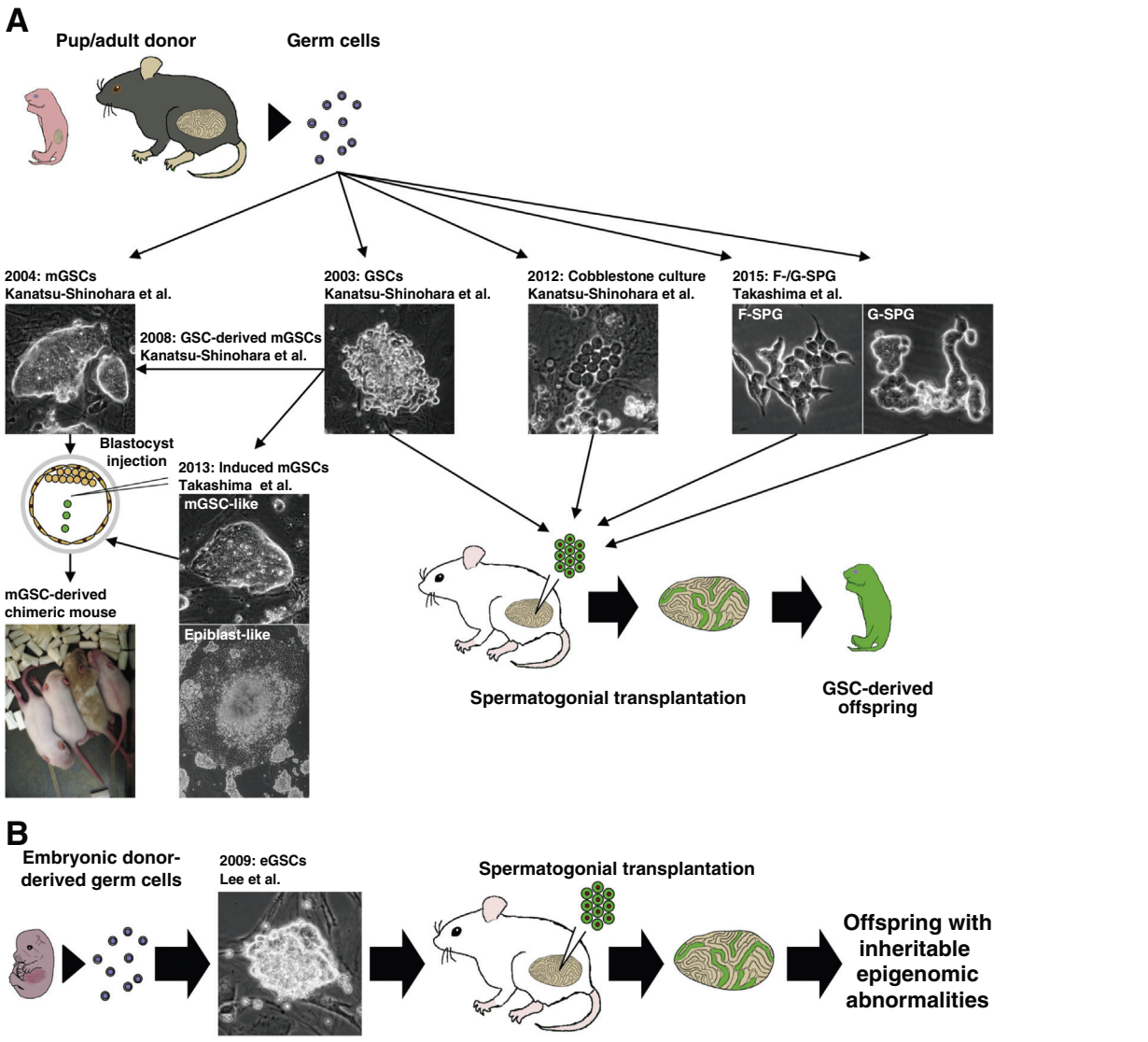

Fig. 3. Development of various culture techniques for SSCs. (A) Stem cell lines from postnatal testes. GSCs form clumps of germ cells on MEFs in the presence of GDNF and FGF2. However, GSCs or SSC-enriched testis cells migrate beneath testis somatic cells to form cobblestone-like colonies. Moreover, F-SPG and G-SPG were also established from pup mouse testes. The former was unexpected because GDNF is believed to be essential for self-renewal. ESC-like mGSCs were found in pup testis cell cultures, suggesting that postnatal germ cells retain potential pluripotency. Although the frequency of mGSC development was low, we found that simultaneous suppression of *Trp53* and *Dmrt1* converts GSCs to mGSCs. These types of ESC-like cells form teratomas following spermatogonial transplantation but contribute to chimeras upon microinjection into blastocysts. (B) eGSCs derived from PGCs. Although eGSCs produce sperm and offspring by spermatogonial transplantation, we found epigenetic abnormalities in the offspring, which were transmitted to subsequent generations.

We focused on the function of FBXW7 in SSC self-renewal because it is specifically expressed in undifferentiated spermatogonia. FBXW7 regulates self-renewal, differentiation, and cell death of various stem cell types (Takeishi and Nakayama, 2014). Using the GSC culture system, we found that overexpression of FBXW7 suppresses GSC proliferation, whereas disruption of *Fbxw7* in SSCs enhances self-renewal and suppresses differentiation. These results showed that FBXW7 negatively influences self-renewal. Screening of FBXW7 target proteins revealed that MYC and CCNE1 are negatively regulated by FBXW7 (Kanatsu-Shinohara et al., 2014b). MYC is particularly important because *Myc*, but not *Ccne1*, overexpression increased colonization of SSCs. Understanding MYC functions will likely lead us to understand the molecular machinery that maintains the balance between self-renewing and differentiating cell divisions.

4. Regulation of self-renewal by the hypothalamic-pituitary (HP) axis

Several previous studies have suggested that SSCs are influenced by the hypothalamic-pituitary (HP) axis. Hypothalamus-derived gonadotropin-releasing hormone (GnRH) stimulates the pituitary gland to release hormones that control gonadal functions. Follicle-stimulating hormone (FSH) acts on Sertoli cells to produce GDNF, while luteinizing hormone (LH) stimulates Leydig cells to synthesize testosterone to regulate Sertoli cells (Lei et al., 2001; Zhang et al., 2001; Tadokoro et al., 2002). The niche size also increases *via* transient hypothyroidism (Oatley et al., 2011). In addition to Leydig cells, peritubular myoid cells (PMCs) produce CSF1 that increases self-renewal *in vitro* (Oatley et al., 2009). PMCs also appear to contribute to SSC self-renewal in humans (Spinnler et al., 2010). Chen et al. recently demonstrated that testosterone-dependent *Gdnf* expression in PMCs contributes to maintenance of undifferentiated spermatogonia *in vitro* and *in vivo* (Chen et al., 2014, 2016). However, the specificity of Cre-mediated deletion in PMCs has been unclear, and it has remained controversial whether testosterone regulates GDNF *in vivo* (Chen and Liu, 2016; Eddy and Chen, 2016).

To clarify the effect of the HP axis on SSCs, we used *Lhcgr* and *Fsh* KO mice (Tanaka et al., 2016). Although FSH reportedly induces GDNF production in Sertoli cells (Tadokoro et al., 2002), we found that FSH does

not influence SSC numbers or expression of GDNF and FGF2 in *Fsh* KO mice. Conversely, the testes of *Lhcgr* KO mice were significantly small and exhibited severe spermatogenic defects. Although we observed relative enrichment of SSCs in *Lhcgr* KO mouse testes, GDNF and FGF2 were not upregulated, suggesting the involvement of unknown self-renewal factors. Therefore, we tried to identify genes responsible for the increased SSC population in *Lhcgr* KO mice using microarray, and found *Wnt5a* as a factor responsible for enhanced self-renewal in *Lhcgr* KO mice. WNT5A was previously shown to improve SSC survival *in vitro* (Yeh et al., 2011). We also confirmed that WNT5A facilitates not only proliferation but also self-renewal of GSCs *in vitro* (Tanaka et al., 2016). Although overexpression of WNT5A in donor testes significantly increases the number of SSCs, overexpression of WNT5A in recipient testes does not affect colonization of donor SSCs. These data demonstrate that WNT5A promotes SSC self-renewal *in vivo* but does not affect SSC homing to their niche (Tanaka et al., 2016).

The involvement of the HP axis in SSC self-renewal was suggested by early spermatogonial transplantation experiments. Colonization efficiency is enhanced by injection of recipient mice with a GnRH analogue (Ogawa et al., 1998; Dobrinski et al., 2001). We also demonstrated that self-renewal efficiency increases when recipients are hypophysectomized before spermatogonial transplantation (Kanatsu-Shinohara et al., 2004b). Although WNT5A stimulates SSC self-renewal, its effect was not as strong as we expected based on these previous experiments, and no effect was found on SSC homing in *Lhcgr* KO mice. Perhaps additional molecules regulate SSC self-renewal and homing under the control of the HP axis.

5. Purification of SSCs

To identify surface markers of SSCs, we have used fluorescence-activated cell sorting and/or magnetic activated cell sorting (MACS) in combination with spermatogonial transplantation. The first surface molecules identified on SSCs were laminin receptors ITGA6 and ITGB1. ITGA6- and ITGB1-mediated selection of SSCs by MACS demonstrated 8.4- and 3.8-fold enrichments of SSCs, respectively (Shinohara et al., 1999). Subsequently, we focused on CD9 because this molecule is expressed on embryonic stem cells (ESCs) that often show a similar gene expression pattern to that of GSCs. Selection of the $CD9^{+}$ population demonstrated a 6.9-fold enrichment of SSCs (Kanatsu-Shinohara et al., 2004c). Epithelial cell adhesion molecule (EPCAM), another molecule expressed in ESCs, is also reportedly useful for enrichment of rat SSCs (Ryu et al., 2004). By combining these two markers, the $CD9^{Hi}EPCAM^{Lo}$ population exhibited a 48.7-fold increase in SSC activity (Kanatsu-Shinohara et al., 2011b). On the other hand, we identified melanoma cell adhesion molecule (MCAM) by screening GSC surface molecules. MCAM labels a broad spectrum of spermatogonia including the KIT^{+} differentiating population but results in only a 2.3-fold higher concentration. However, MCAM expression in GSCs depends on self-renewal factors, suggesting that MCAM expression reflects the magnitude of GDNF/FGF2 signals. The $CD9^{Hi}EPCAM^{Lo}KIT^{-}MCAM^{+}$ population achieved a 560.5-fold higher concentration of functional SSCs. The frequency of SSCs in this population was one in six cells (Kanatsu-Shinohara et al., 2012b).

Stem cell dyes are also applicable for SSC enrichment. Although Hoechst 33342 staining revealed that hematopoietic stem cells (HSCs) are resided in the side population, several groups reported conflicting results of SSC activity in the testicular side population: Some groups achieved SSCs enrichment, while other groups failed to find SSCs in the side population (Kubota et al., 2003; Falciatori et al., 2004; Lassalle et al., 2004; Lo et al., 2005; Barroca et al., 2009; Shinohara et al., 2011). At present, it is difficult to reconcile these observations. However, one possibility is the type of donor testes. SSCs in cryptorchid testes may not necessarily have the same properties with those in untreated testes (Lassalle et al., 2004). Moreover, because Hoecsht 33342 exhibit cell toxicity, slight difference in Hoechst concentration might have destroyed SSCs (Lo et al., 2005).

By contrast, activity of aldehyde dehydrogenase (ALDH) was found to be useful for somatic stem cell isolation (Tomita et al., 2016). ALDHs are a family of enzymes that detoxify aldehyde moieties by oxidation (Balber, 2011), and can be detected by exposing cells to boron-dipyrromethene-conjugated aminoacetaldehyde. We have applied this procedure to concentrate SSCs from $CD9^{+}$- or $CDH1^{+}$ testis cells and achieved 22.2- and 186.0-fold enrichments of SSCs, respectively. In contrast to our expectation, SSCs were enriched in the $ALDH^{-}$ population (Kanatsu-Shinohara et al., 2013). However, there are still some aspects that need improvement. First, ALDH-mediated selection is not specific to SSCs in culture. Another problem is that ALDH activity does not positively select SSCs. Considering that stem cells in other tissues are $ALDH^{+}$ populations, this result was unexpected. Because the $ALDH^{-}$ population contains somatic cells, it is difficult to distinguish SSCs from contaminating somatic cells, which decreases the efficiency of enrichment.

To overcome this problem, we next used fluorescent molecules CDy1 and rhodamine 123 (Rh123) (Kanatsu-Shinohara et al., 2016a). CDy1 is a substrate of ATP-binding cassette sub-family B member 1B and can distinguish undifferentiated ESCs/induced pluripotent stem cells (iPSCs) from differentiated progenies in culture (Vendrell et al., 2012). On the other hand, Rh123 is a fluorescent substrate for P-glycoprotein and is reported to be applicable to SSC enrichment from cryptorchid testes by collection of the $Rh123^{Lo}$ population (Lo et al., 2005). Our transplantation assay demonstrated that CDy1 staining positively detects SSCs not only in GSC cultures but also among $CD9^{+}$- or $CDH1^{+}$-selected testis cells: $CDy1^{High}$ GS cells exhibited 2.1-fold enrichment of SSCs compared to unfractionated population, while $CDy1^{High}$ $CD9^{+}$ population and $CDH1^{+}$ population of adult mouse testis cells exhibited 64.5-fold and 204.0-fold enrichment of SSCs compared to unfractionated testis cells. Conversely, Rh123 staining was not useful for SSC enrichment. The difference between wild-type and cryptorchid testes may be caused by the higher temperature in cryptorchid testes, but other factors may be involved (Kanatsu-Shinohara et al., 2016a). Because dye-based SSC selection does not rely on species-specific antibodies, this procedure may be applicable to other animal species for which appropriate antibodies for SSC isolation are unavailable.

Based on these analyses in rodents, several groups tried to purify human SSCs. Especially, ITGA6 and CD9 was suggested to be useful marker for human SSC purification by MACS in combination with spermatogonial transplantation into testes of germ cell-depleted nude mice (Zohni et al., 2012; Valli et al., 2014). It would be important to examine whether other putative SSC markers found in rodents are conserved in human SSCs.

6. Homing of SSCs to the germline niche

Migration of transplanted SSCs through the blood testis barrier (BTB) into niches is probably the most striking observation in spermatogonial transplantation, because spermatogenic cells in the basal compartment of seminiferous tubules normally migrate to the adluminal compartment. This SSC “homing” phenomenon does not appear to be a physiological event. However, it underscores the close relationship between SSCs and niches. Although the molecular mechanism of SSC homing had been unknown, we identified ITGB1 as a critical molecule for SSC homing in 2008. Transplanted SSCs lacking this molecule migrate to the basal compartment of seminiferous tubules, but they detach from the basement membrane and fail to form germ cell colonies (Kanatsu-Shinohara et al., 2008d). In 2011, RAC1 was demonstrated to be an essential molecule for passing through the BTB (Takashima et al., 2011). Although SSCs lacking RAC1 hardly colonize the adult germline niche, the same cells colonize pup testes with an immature BTB. RAC1 is considered to regulate CLDN3 to pass through the tight junctions of the BTB. SSC homing also depends on the cell cycle status,

because GSCs in G1 phase produce significantly more colonies than those in other cell cycle phases (Ishii et al., 2014b).

We established a novel *in vitro* homing assay for SSCs using testis cell feeders. This assay is somewhat similar to the cobblestone area-forming assay for HSCs (Kanatsu-Shinohara et al., 2012a). HSCs form cobblestone-like colonies beneath a bone marrow feeder layer (Ploemacher et al., 1989), whereas ITGA6^{+} germ cells enriched for SSCs and GSCs formed such colonies under feeder layer made from testicular somatic cells derived from *Kit*^{W}/*Kit*$^{W\text{-}v}$ (W) mice (Fig. 3A). These results suggest that the cobblestone-forming activity is conserved between HSCs and SSCs.

Using this assay, we found that GDNF and CXCL12 are involved in SSC homing. These results are consistent with other reports (Dovere et al., 2013; Huleihel et al., 2013; Yang et al., 2013b). Both GDNF and CXCL12 enhance cobblestone area formation by SSCs and GSCs *in vitro*, whereas inhibition of these signals reduce the homing efficiency after spermatogonial transplantation, demonstrating that they contribute to SSC homing *in vivo*. Because GDNF increases the expression of CXCR4, a receptor for CXCL12, it is likely that these factors act synergistically for SSC homing.

7. Potential pluripotency of SSCs

During the course of gene targeting experiments using GSCs, ESC-like colonies were found in pup testis cell cultures. These cells were designated as multipotent GSCs (mGSCs) (Fig. 3A). They showed *bona fide* pluripotency and contributed to germline chimeras by blastocyst injection (Kanatsu-Shinohara et al., 2004a). This result was unexpected because it has been considered that germ cells lose pluripotency after 13.5 days postcoitum (dpc). Leroy C. Stevens reported that male genital ridges from 129/Sv mouse at 11.5–12.5 dpc frequently formed teratomas in the recipient adult testes. However, this incidence was dramatically decreased when genital ridges were collected from 13.5 dpc or older embryos (Stevens, 1966). Moreover, primordial germ cells (PGCs) derived from 8.5 to 12.5 dpc embryos give rise to embryonic germ cells under specialized culture conditions containing KITL, FGF2, and leukemia inhibitory factor. However, the same culture conditions do not induce pluripotency when cells are collected from 13.5 dpc or older embryos (Matsui et al., 1992; Resnick et al., 1992; Labosky et al., 1994). Although it was possible that a small number of primitive PGC-like cells remained in postnatal testes, it was likely that mGSCs originated from GSCs because we found that GSCs and mGSCs with the same transgene integration patterns appeared during drug selection of transfected clones after electroporation (Kanatsu-Shinohara et al., 2008a). These data demonstrated that mGSCs originate from GSCs. Discovery of mGSCs suggested that postnatal germ cells retain potential pluripotency.

The involvement of *Trp53* in mGSC formation was a by-product of our research on radiation sensitivity (Ishii et al., 2014a). When we tried to establish GSCs from *Trp53* KO mice, we noticed that *Trp53* KO GSCs readily convert into mGSCs (Kanatsu-Shinohara et al., 2004a). Because *Trp53* is an established tumor suppressor gene, this observation led us to analyze the relationship between mGSCs and germ cell tumor formation. Although HRASV12 and CCND2 have been implicated in germ cell tumor development, they do not facilitate mGSC derivation (Lee et al., 2009a). We next applied an *in vitro* transformation protocol developed for somatic cells. Pup testis cells were transfected with HRASV12, MYC, and dominant negative TRP53. Similar to ESCs, the combination of these genes produced ESC-like colonies that proliferated very rapidly. However, they did not express *Nanog*, a critical marker of pluripotency (Morimoto et al., 2012). Interestingly, transfecting the same combination of genes or Yamanaka factors did not induce apparent changes in GSCs, suggesting that they are resistant to somatic cell transformation/reprogramming protocols. These results indicate that simple overexpression of somatic cell oncogenes does not induce mGSCs from fresh SSCs, and suggest that GSCs and fresh testis cells may not behave similarly, which supports our previous experiments that showed phenotypic differences between GSCs and SSCs *in vivo*. For example, it is generally considered that SSCs *in vivo* do not express KIT. However, both KIT^{+} and KIT^{-} populations in GSC cultures exhibit comparable SSC frequency (Morimoto et al., 2009). Perhaps, such difference may be caused by the excessive stimulation of self-renewal *in vitro*.

We then applied different approach. Because imprinted genes are demethylated in mGSCs, we hypothesized that DNA demethylation might play a role during dedifferentiation of GSCs (Kanatsu-Shinohara et al., 2004a). Moreover, the degree of genome-wide CpG methylation in GSCs is significantly higher than that in mGSCs and ESCs (Takashima et al., 2013). Based on these observations, we depleted *Dnmt1* and *Trp53* in GSCs and found that simultaneous suppression of these genes induces mGSCs from GSCs within 4 weeks at an efficiency of 70%. Therefore, DNA methylation is important for suppression of pluripotency in GSCs. Subsequently, we identified *Dmrt1* as a gene that regulates potential pluripotency. *Dmrt1* is downregulated in *Trp53* KO GSCs upon *Dnmt1* knockdown, and simultaneous depletion of *Dmrt1* and *Trp53* induces pluripotency in GSCs *via* upregulation of *Sox2* (Takashima et al., 2013) (Fig. 3A). Moreover, we demonstrated that *Sox2* upregulation induces *Pou5f1*. Although *Dmrt1* is responsible for sex determination and meiosis, deletion of this gene also induces teratomas at an efficiency of 90% in the 129/Sv strain (Krentz et al., 2009; Matson et al., 2010; Matson et al., 2011). These data suggest that *Dmrt1* is a germline-specific tumor suppressor gene and one of the critical molecules that suppress pluripotency in GSCs.

However, there were more questions raised by these experiments. We recently found that depletion of *Trp53* and *Dmrt1* does not induce pluripotency in freshly isolated testis cells. In addition, although we assumed that younger germ cells would be more sensitive to induction of pluripotency, *in vitro* transfection experiments revealed that PGCs derived from 12.5 dpc embryo are more sensitive than those from 8.5 to 10.5 dpc embryos. *Dmrt1* suppression does not appear to be necessary to induce pluripotent cells because depleting *Trp53* suppression is sufficient for conversion (Tanaka et al., 2015). These experiments indicate that 1) PGCs/SSCs/GSCs do not show the same phenotypes upon *Dmrt1*/*Trp53* depletion, 2) *Trp53* is critical to suppress pluripotency throughout germline development, and 3) *Dmrt1* plays an additional role in suppression of pluripotency in postnatal germ cells. Germ cells are unique because all Yamanaka factors are expressed at the mRNA level (Takashima et al., 2013). However, why primary germ cells do not become pluripotent cells is not entirely clear. Because simple transfection of Yamanaka factors does not induce iPSCs from GSCs, germ cells including SSCs might have a unique machinery to regulate pluripotency, which does not operate in somatic cells.

8. SSCs and fertility

The most stringent criterion of SSC functions is fertility restoration. Although spermatogenesis may appear normal according to histology after transplantation, it does not necessarily indicate that SSCs are functionally normal. In fact, GSC-like cells that originate from PGC cultures (embryonic GSCs: eGSCs) show abnormal DNA methylation in offspring (Lee et al., 2009b) (Fig. 3B). However, few studies have addressed basic questions associated with fertility restoration, possibly because of the difficulties and inefficiency of the spermatogonial transplantation technique.

Initial transplantation experiments showed the feasibility of fertility restoration. However, the efficiency of producing offspring was very low; three recipients had restored fertility at least 8 months after spermatogonial transplantation (Brinster and Avarbock, 1994) (Fig. 1). The use of W mice increased the efficiency, but more than 5 months were still required to produce offspring (Ogawa et al., 2000). The most dramatic improvement was achieved using immature W mice (Shinohara et al., 2001). Because the BTB would be a big barrier for SSC colonization, we hypothesized that pup testes without the BTB increase SSC

colonization. As expected, the efficiency of donor cell colonization in these mice increases by 5–10-fold, and colonies are significantly larger than those in adult mice. Most importantly, offspring are produced within 3 months after transplantation. It is considered that the increased colonization is due to the lack of the BTB in these mice. In addition, immature Sertoli cells might have characteristics more hospitable for SSCs by secreting significantly higher levels of GDNF (Meng et al., 2000). This transplantation technique has significantly improved fertility studies and has been used to produce offspring from not only SSCs but also PGCs (Chuma et al., 2005; Hayashi et al., 2011), which do not colonize adult testes (Ohta et al., 2004).

One of the questions raised in our fertility restoration experiments was how the genetic information is transmitted to the next generation. Using KO mice, we found that cyclin-dependent kinase (CDK) inhibitors affect the contribution efficiency of SSC to fertility (Kanatsu-Shinohara et al., 2010). The cell cycle is positively regulated by CDK and cyclin, whereas CDK inhibitors regulate the cell cycle negatively by inducing cell cycle exit *via* inhibiting the formation of the cyclin-CDK complex. We focused on the function of two CDK inhibitors, *Cdkn1a* and *Cdkn1b*. It has been reported that *Cdkn1b*-deficient mice exhibit multiorgan hyperplasia, including in the testis, which was attributed to Sertoli cell defects (Nakayama et al., 1997). Conversely, *Cdkn1a*-deficient mice do not show apparent testicular abnormalities under physiological conditions (Deng et al., 1995). However, when the same number of *Cdkn1b*-deficient and wild-type testis cells were injected into W mice to compare the efficiency of germline transmission (competitive repopulation assay), we found that *Cdkn1b*-deficient SSCs rarely contribute to offspring. In contrast, when we performed similar experiments using *Cdkn1a* KO mouse testis cells, SSCs from *Cdkn1a* KO mice outcompeted wild-type SSCs. Although the exact mechanisms underlying such phenomena are unknown, these results suggest that the amount of CDK inhibitors influences the germline transmission patterns in the male.

We further analyzed the germline transmission pattern of individual SSCs (Kanatsu-Shinohara et al., 2016c). SSCs are thought to contribute randomly to fertilization events. However, we found that a small number of SSCs produce offspring within a set period. Interestingly, the same SSC clone reappears later with an average functional life span of ~124.4 days. This cyclic offspring production pattern does not depend on the mode of self-renewal because our lineage tracing analysis revealed that all SSCs proliferate continuously. Selection of SSC clones appears to occur during the differentiating spermatogonia stage during which extensive apoptosis was detected. These data suggest that, although all SSC clones evenly undergo self-renewal and produce differentiating spermatogonia, the progeny of some SSC clones contribute to fertility at a given time point (Kanatsu-Shinohara et al., 2016c). The dynamics of offspring production from SSCs can be predicted using a mathematical model in which SSCs repeat cycles of transient spermatogenic burst and refractory periods, suggesting that spermatogenesis is a regulated process through which specific SSC clones take turns to contribute to fertility with a long-term cycle.

Fertility studies using W pup recipients have several problems including the technical difficulties of transplantation and availability of mutant male mice. To overcome these problems, we recently reported the utility of GSCs and adult recipients for fertility studies (Fig. 1). Because GSCs are enriched for SSCs, it allows us to transplant a large number of SSCs for fertility restoration. In addition, spermatogonial transplantation into adult testes is more reliable than using pup testes. In this experiment, we compared the utility of busulfan-treated wild-type adult (Bus) and congenitally infertile adult W mice (Kanatsu-Shinohara et al., 2016b). Despite several previous reports showing the superiority of W mice, we found efficient production of offspring by Bus recipients. All seven Bus recipients produced donor-derived offspring within 4 months after transplantation. In contrast, three of five W recipients became fertile. Quantification of SSCs revealed that at least 40–80 SSCs are required to restore fertility. This fertility restoration system using GSCs and Bus mice will be useful to understand factors involved in male germline transmission.

9. SSC applications

GSCs are potentially useful for genetic manipulation of a variety of animals including humans. However, several obstacles need to be overcome.

Optimization of the GSC culture technique is apparently necessary. After development of mouse GSC culture, a similar culture technique was developed for rats and hamsters (Ryu et al., 2005; Hamra et al., 2005; Kanatsu-Shinohara et al., 2008b). However, long-term cultures for more than months have not been achieved for SSCs from non-rodent species. Even in rodents, the behavior of the cultured cells is quite variable. For example, although rat SSCs proliferate faster than mouse SSCs *in vivo*, rat GSCs are unstable and proliferate more slowly than mouse GSCs (Orwig et al., 2002; Kanatsu-Shinohara et al., 2011a). Similarly, although Kubota et al. succeeded in expanding rabbit spermatogonia *in vitro*, the stem cell activity of the cultured cells was diminished severely, suggesting that the cells gradually lose SSC activity during culture (Kubota et al., 2011). These results suggest that the current culture conditions are far from ideal. Although clustered regularly interspaced short palindromic repeat-CRISPR-associated protein 9 (CRISPR-Cas9) technology enables more efficient genetic manipulation in rodents (Yang et al., 2013a; Chapman et al., 2015; Sato et al., 2015b; Wu et al., 2015) (Fig. 2B), it is apparently necessary to identify novel self-renewal factors to extend the technique to a large number of animal species. Development of serum-free culture techniques will be useful to resolve this issue, which are being improved for mice (Kanatsu-Shinohara and Shinohara, 2013; Kanatsu-Shinohara et al., 2014a).

Another critical problem in SSC applications is improvement of the transplantation technique. The spermatogonial transplantation technique was originally developed in mice and subsequently extended to rats (Brinster and Zimmermann, 1994; Ogawa et al., 1999). Although SSC transplantation was applied to many non-rodent animals, SSC-derived offspring were born only in goat, sheep, and chicken (Fig. 1) (Honaramooz et al., 2003; Lee et al., 2006; Trefil et al., 2006; Herrid et al., 2009). Two factors are involved in fertility restoration using spermatogonial transplantation. The first is the number of SSCs. Transplantation of fresh testis cells is apparently inefficient because SSCs are only a small population in testes. As mentioned above, transplantation of GSCs is more practical because it is possible to collect a large number of SSCs (Kanatsu-Shinohara et al., 2016a). Therefore, derivation of GSC cultures will likely solve this first problem. Another factor is recipient preparation. Removal of endogenous germ cells compromises testis microenvironments in rats (Ogawa et al., 1999). In addition, differences in testicular structures need to be taken into consideration. A much larger number of cells might be required for fertility restoration because of their large testis size. However, microinsemination technique can be used to produce offspring from transplanted SSCs (Kanatsu-Shinohara et al., 2003b; Shinohara et al., 2006) (Fig. 1).

In this context, the *in vitro* spermatogenesis technique will be an attractive approach to solve the second problem. Sato et al. applied the liquid-air interface type organ culture technique and succeeded in inducing *in vitro* spermatogenesis of seminiferous tubules derived not only from pups but also several types of mice, including wild-type adult, $Kitl^{Sl}/Kitl^{Sl\text{-}d}$ (Sl) mutant, and cryopreserved pup testes (Sato et al., 2011a, 2012; Yokonishi et al., 2014; Sato et al., 2015a) (Fig. 4). Moreover, Komeya et al. improved the efficiency of spermatogenesis by adopting microfluidics technology (Komeya et al., 2016). Although these studies suggest promising future outcomes, the next challenge is to extend this technique to other animal species. Because GSC transplantation into cultured testicular fragments is clearly difficult and requires skilled handling (Sato et al., 2011b), the final goal is to induce GSCs to differentiate into sperm in two-dimensional culture without somatic cells.

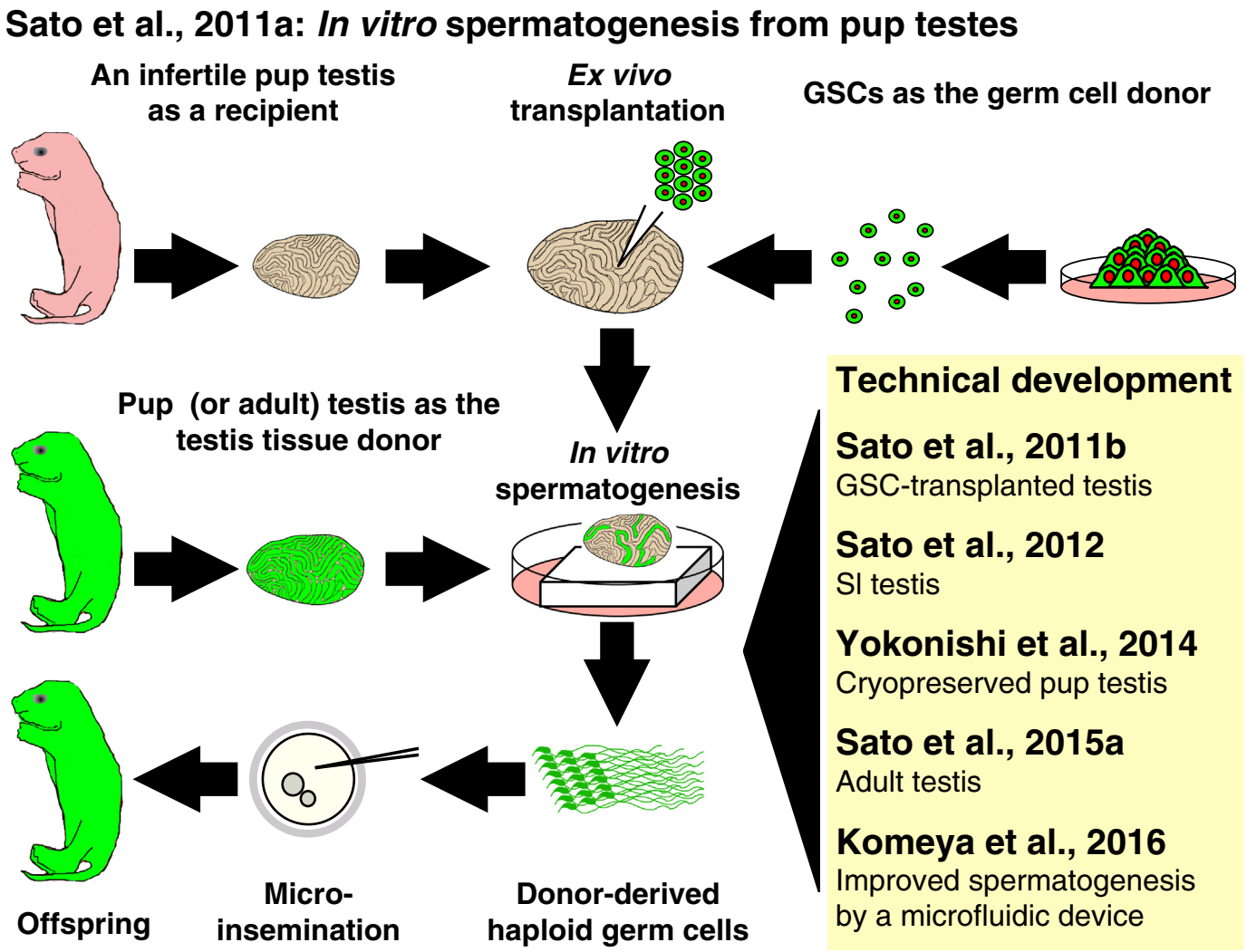

Fig. 4. Development of the *in vitro* spermatogenesis technique using an organ culture system. Sato et al. achieved *in vitro* spermatogenesis using mouse pup testes. This technique was subsequently adapted to produce offspring from GSCs, cryopreserved testes, and adult testes. They also demonstrated fertility restoration of SI testes by an overload of KITL and succeeded to improve the efficiency of spermatogenesis using a microfluidics system.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudging the impartiality of the reported research.

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References

Balber, A.E., 2011. Concise review: aldehyde dehydrogenase bright stem and progenitor cell populations from normal tissues: characteristics, activities, and emerging uses in regenerative medicine. Stem Cells 29, 570–575.

Barroca, V., Lassalle, B., Coureuil, M., Louis, J.P., Le Page, F., Testart, J., Allemand, I., Riou, L., Fouchet, P., 2009. Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nat. Cell Biol. 11, 190–196.

Brinster, R.L., Avarbock, M.R., 1994. Germline transmission of donor haplotype following spermatogonial transplantation. Proc. Nat. Acad. Sci. U. S. A. 91, 11303–11307.

Brinster, R.L., Zimmermann, J.W., 1994. Spermatogenesis following male germ-cell transplantation. P Proc. Nat. Acad. Sci. U. S. A. 91, 11298–11302.

Chapman, K.M., Medrano, G.A., Jaichander, P., Chaudhary, J., Waits, A.E., Nobrega, M.A., Hotaling, J.M., Ober, C., Hamra, F.K., 2015. Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. Cell Rep. 10, 1828–1835.

Chen, S.R., Liu, Y.X., 2016. *Myh11*-Cre is not limited to peritubular myoid cells and interaction between Sertoli and peritubular myoid cells needs investigation. Proc. Natl. Acad. Sci. U. S. A. 113, E2352.

Chen, L.Y., Brown, P.R., Willis, W.B., Eddy, E.M., 2014. Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinol. 155, 4964–4974.

Chen, L.Y., Willis, W.D., Eddy, E.M., 2016. Targeting the *Gdnf* gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc. Natl. Acad. Sci. U. S. A. 113, 1829–1834.

Chuma, S., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Hosokawa, M., Nakatsuji, N., Ogura, A., Shinohara, T., 2005. Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. Development 132, 117–122.

de Rooij, D.G., Russell, L.D., 2000. All you wanted to know about spermatogonia but were afraid to ask. J. Androl. 21, 776–798.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., Leder, P., 1995. Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. Cell 82, 675–684.

Dobrinski, I., Ogawa, T., Avarbock, M.R., Brinster, R.L., 2001. Effect of the GnRH-agonist leuprolide on colonization of recipient testes by donor spermatogonial stem cells after transplantation in mice. Tissue Cell 33, 200–207.

Dovere, L., Fera, S., Grasso, M., Lamberti, D., Gargioli, C., Muciaccia, B., Lustri, A.M., Stefanini, M., Vicini, E., 2013. The niche-derived glial cell line-derived neurotrophic factor (GDNF) induces migration of mouse spermatogonial stem/progenitor cells. PLoS One 8, e59431.

Eddy, E.M., Chen, L.Y., 2016. Reply to Chen and Liu: role of GDNF from peritubular myoid cells in the testis stem cell niche. Proc. Natl. Acad. Sci. U. S. A. 113, E2353.

Falciatori, I., Borsellino, G., Haliassos, N., Boitani, C., Corallini, S., Battistini, L., Bernardi, G., Stefanini, M., Vicini, E., 2004. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J. 18, 376–378.

Goriely, A., Wilkie, A.O., 2012. Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. Am. J. Hum. Genet. 90, 175–200.

Hamra, F.K., Gatlin, J., Chapman, K.M., Grellhesl, D.M., Garcia, J.V., Hammer, R.E., Garbers, D.L., 2002. Production of transgenic rats by lentiviral transduction of male germline stem cells. Proc. Natl. Acad. Sci. U. S. A. 99, 14931–14936.

Hamra, F.K., Chapman, K.M., Nguyen, D.M., Williams-Stephens, A.A., Hammer, R.E., Garbers, D.L., 2005. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc. Natl. Acad. Sci. U. S. A. 102, 17430–17435.

Hasegawa, K., Saga, Y., 2014. FGF8-FGFR1 signaling acts as a niche factor for maintaining undifferentiated spermatogonia in the mouse. Biol. Reprod. 91, 145.

Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., Saitou, M., 2011. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 146, 519–532.

Herrid, M., Davey, R.J., Hutton, K., Colditz, I.G., Hill, J.R., 2009. A comparison of methods for preparing enriched populations of bovine spermatogonia. Reprod. Fertil. Dev. 21, 393–399.

Honaramooz, A., Behboodi, E., Megee, S.O., Overton, S.A., Galantino-Homer, H., Echelard, Y., Dobrinski, I., 2003. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol. Reprod. 69, 1260–1264.

Huleihel, M., Fadlon, E., Abuelhija, A., Piltcher Haber, E., Lunenfeld, E., 2013. Glial cell line-derived neurotrophic factor (GDNF) induced migration of spermatogonial cells in vitro via MEK and NF-kB pathways. Differentiation 86, 38–47.

Ishii, K., Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T., 2012. FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of *Etv5* and *Bcl6b* through MAP2K1 activation. Development 139, 1734–1743.

Ishii, K., Ishiai, M., Morimoto, H., Kanatsu-Shinohara, M., Niwa, O., Takata, M., Shinohara, T., 2014a. The *Trp53-Trp53inp1-Tnfrsf10b* pathway regulates the radiation response of mouse spermatogonial stem cells. Stem Cell Rep. 3, 676–689.

Ishii, K., Kanatsu-Shinohara, M., Shinohara, T., 2014b. Cell-cycle-dependent colonization of mouse spermatogonial stem cells after transplantation into seminiferous tubules. J. Reprod. Dev. 60, 37–46.

Jain, S., Naughton, C.K., Yang, M., Strickland, A., Vij, K., Encinas, M., Golden, J., Gupta, A., Heuckeroth, R., Johnson Jr., E.M., Milbrandt, J., 2004. Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. Development 131, 5503–5513.

Jijiwa, M., Kawai, K., Fukihara, J., Nakamura, A., Hasegawa, M., Suzuki, C., Sato, T., Enomoto, A., Asai, N., Murakumo, Y., Takahashi, M., 2008. GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells 13, 365–374.

Kanatsu-Shinohara, M., Shinohara, T., 2013. Spermatogonial stem cell self-renewal and development. Annu. Rev. Cell Dev. Biol. 29, 163–187.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., Shinohara, T., 2003a. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. 69, 612–616.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Ogura, A., Toyokuni, S., Honjo, T., Shinohara, T., 2003b. Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. Biol. Reprod. 68, 167–173.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., et al., 2004a. Generation of pluripotent stem cells from neonatal mouse testis. Cell 119, 1001–1012.

Kanatsu-Shinohara, M., Morimoto, T., Toyokuni, S., Shinohara, T., 2004b. Regulation of mouse spermatogonial stem cell self-renewing division by the pituitary gland. Biol. Reprod. 70, 1731–1737.

Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T., 2004c. CD9 is a surface marker on mouse and rat male germline stem cells. Biol. Reprod. 70, 70–75.

Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T., 2004d. Transgenic mice produced by retroviral transduction of male germ line stem cells in vivo. Biol. Reprod. 71, 1202–1207.

Kanatsu-Shinohara, M., Ogonuki, N., Iwano, T., Lee, J., Kazuki, Y., Inoue, K., Miki, H., Takehashi, M., Toyokuni, S., Shinkai, Y., Oshimura, M., Ishino, F., Ogura, A., Shinohara, T., 2005a. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132, 4155–4163.

Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T., 2005b. Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells. Biol. Reprod. 72, 236–240.

Kanatsu-Shinohara, M., Ikawa, M., Takehashi, M., Ogonuki, N., Miki, H., Inoue, K., Kazuki, Y., Lee, J., Toyokuni, S., Oshimura, M., et al., 2006a. Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc. Natl. Acad. Sci. U. S. A. 103, 8018–8021.

Kanatsu-Shinohara, M., Inoue, K., Miki, H., Ogonuki, N., Takehashi, M., Morimoto, T., Ogura, A., Shinohara, T., 2006b. Clonal origin of germ cell colonies after spermatogonial transplantation in mice. Biol. Reprod. 75, 68–74.

Kanatsu-Shinohara, M., Lee, J., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Ikawa, M., Nakamura, T., Ogura, A., Shinohara, T., 2008a. Pluripotency of a single spermatogonial stem cell in mice. Biol. Reprod. 78, 681–687.

Kanatsu-Shinohara, M., Muneto, T., Lee, J., Takenaka, M., Chuma, S., Nakatsuji, N., Horiuchi, T., Shinohara, T., 2008b. Long-term culture of male germline stem cells from hamster testes. Biol. Reprod. 78, 611–617.

Kanatsu-Shinohara, M., Takehashi, M., Shinohara, T., 2008c. Brief history, pitfalls, and prospects of mammalian spermatogonial stem cell research. Cold Spring Harb. Symp. Quant. Biol. 73, 17–23.

Kanatsu-Shinohara, M., Takehashi, M., Takashima, S., Lee, J., Morimoto, H., Chuma, S., Raducanu, A., Nakatsuji, N., Fässler, R., Shinohara, T., 2008d. Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell 3, 533–542.

Kanatsu-Shinohara, M., Takashima, S., Shinohara, T., 2010. Transmission distortion by loss of p21 or p27 cyclin-dependent kinase inhibitors following competitive spermatogonial transplantation. Proc. Natl. Acad. Sci. U. S. A. 107, 6210–6215.

Kanatsu-Shinohara, M., Kato-Itoh, M., Ikawa, M., Takehashi, M., Sanbo, M., Morioka, Y., Tanaka, T., Morimoto, H., Hirabayashi, M., Shinohara, T., 2011a. Homologous recombination in rat germline stem cells. Biol. Reprod. 85, 208–217.

Kanatsu-Shinohara, M., Takashima, S., Ishii, K., Shinohara, T., 2011b. Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6, e23663.

Kanatsu-Shinohara, M., Inoue, K., Takashima, S., Takehashi, M., Ogonuki, N., Morimoto, H., Nagasawa, T., Ogura, A., Shinohara, T., 2012a. Reconstitution of mouse spermatogonial stem cell niches in culture. Cell Stem Cell 11, 567–578.

Kanatsu-Shinohara, M., Morimoto, H., Shinohara, T., 2012b. Enrichment of mouse spermatogonial stem cells by melanoma cell adhesion molecule expression. Biol. Reprod. 87, 139.

Kanatsu-Shinohara, M., Mori, Y., Shinohara, T., 2013. Enrichment of mouse spermatogonial stem cells based on aldehyde dehydrogenase activity. Biol. Reprod. 89, 140.

Kanatsu-Shinohara, M., Ogonuki, N., Matoba, S., Morimoto, H., Ogura, A., Shinohara, T., 2014a. Improved serum- and feeder-free culture of mouse germline stem cells. Biol. Reprod. 91, 88.

Kanatsu-Shinohara, M., Onoyama, I., Nakayama, K.I., Shinohara, T., 2014b. Skp1-Cullin-F-box (SCF)-type ubiquitin ligase FBXW7 negatively regulates spermatogonial stem cell self-renewal. Proc. Natl. Acad. Sci. U. S. A. 111, 8826–8831.

Kanatsu-Shinohara, M., Morimoto, H., Shinohara, T., 2016a. Enrichment of mouse spermatogonial stem cells by the stem cell dye CDy1. Biol. Reprod. 94, 13.

Kanatsu-Shinohara, M., Morimoto, H., Shinohara, T., 2016b. Fertility of male germline stem cells following spermatogonial transplantation in infertile mouse models. Biol. Reprod. 94, 112.

Kanatsu-Shinohara, M., Naoki, H., Shinohara, T., 2016c. Nonrandom germline transmission of mouse spermatogonial stem cells. Dev. Cell 38, 248–261.

Komeya, M., Kimura, H., Nakamura, H., Yokonishi, T., Sato, T., Kojima, K., Hayashi, K., Katagiri, K., Yamanaka, H., Sanjo, H., et al., 2016. Long-term *ex vivo* maintenance of testis tissues producing fertile sperm in a microfluidic device. Sci. Rep. 6, 21472.

Krentz, A.D., Murphy, M.W., Kim, S., Cook, M.S., Capel, B., Zhu, R., Matin, A., Sarver, A.L., Parker, K.L., Griswold, M.D., et al., 2009. The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. Proc. Natl. Acad. Sci. U. S. A. 106, 22323–22328.

Kubota, H., Avarbock, M.R., Brinster, R.L., 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc. Natl. Acad. Sci. U. S. A. 100, 6487–6492.

Kubota, H., Avarbock, M.R., Brinster, R.L., 2004. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. U. S. A. 101, 16489–16494.

Kubota, H., Wu, X., Goodyear, S.M., Avarbock, M.R., Brinster, R.L., 2011. Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J. 25, 2604–2614.

Labosky, P.A., Barlow, D.P., Hogan, B.L., 1994. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (*Igf2r*) gene compared with embryonic stem (ES) cell lines. Development 120, 3197–3204.

Lassalle, B., Bastos, H., Louis, J.P., Riou, L., Testart, J., Dutrillaux, B., Fouchet, P., Allemand, I., 2004. Side population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 131, 479–487.

Lee, Y.M., Jung, J.G., Kim, J.N., Park, T.S., Kim, T.M., Shin, S.S., Kang, D.K., Lim, J.M., Han, J.Y., 2006. A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol. Reprod. 75, 380–386.

Lee, J., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Kimura, T., Nakano, T., Ogura, A., Shinohara, T., 2007. Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134, 1853–1859.

Lee, J., Kanatsu-Shinohara, M., Morimoto, H., Kazuki, Y., Takashima, S., Oshimura, M., Toyokuni, S., Shinohara, T., 2009a. Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation. Cell Stem Cell 5, 76–86.

Lee, J., Kanatsu-Shinohara, M., Ogonuki, N., Miki, H., Inoue, K., Morimoto, T., Morimoto, H., Ogura, A., Shinohara, T., 2009b. Heritable imprinting defect caused by epigenetic abnormalities in mouse spermatogonial stem cells. Biol. Reprod. 80, 518–527.

Lei, Z.M., Mishra, S., Zou, W., Xu, B., Foltz, M., Li, X., Rao, C.V., 2001. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol. Endocrinol. 15, 184–200.

Lo, K.C., Brugh, V.M.I.I.I., Parker, M., Lamb, D.J., 2005. Isolation and enrichment of murine spermatogonial stem cells using rhodamine 123 mitochondrial dye. Biol. Reprod. 72, 767–771.

Lu, N., Sargent, K.M., Clopton, D.T., Pohlmeier, W.E., Brauer, V.M., McFee, R.M., Weber, J.S., Ferrara, N., Silversides, D.W., Cupp, A.S., 2013. Loss of vascular endothelial growth factor A (VEGFA) isoforms in the testes of male mice causes subfertility, reduces sperm numbers, and alters expression of genes that regulate undifferentiated spermatogonia. Endocrinology 154, 4790–4802.

Matson, C.K., Murphy, M.W., Griswold, M.D., Yoshida, S., Bardwell, V.J., Zarkower, D., 2010. The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. Dev. Cell 19, 612–624.
Matson, C.K., Murphy, M.W., Sarver, A.L., Griswold, M.D., Bardwell, V.J., Zarkower, D., 2011. DMRT1 prevents female reprogramming in the postnatal mammalian testis. Nature 476, 101–104.
Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841–847.
Meistrich, M.L., van Beek, M.E.A.B., 1993. Spermatogonial stem cells. Cell Mol. Biol. Testis, 266–295.
Meng, X., Lindahl, M., Hyvönen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., et al., 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287, 1489–1493.
Morimoto, H., Kanatsu-Shinohara, M., Takashima, S., Chuma, S., Nakatsuji, N., Takehashi, M., Shinohara, T., 2009. Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One 4, e7909.
Morimoto, H., Lee, J., Tanaka, T., Ishii, K., Toyokuni, S., Kanatsu-Shinohara, M., Shinohara, T., 2012. In vitro transformation of mouse testis cells by oncogene transfection. Biol. Reprod. 86, 148.
Morimoto, H., Iwata, K., Ogonuki, N., Inoue, K., Atsuo, O., Kanatsu-Shinohara, M., Morimoto, T., Yabe-Nishimura, C., Shinohara, T., 2013. ROS are required for mouse spermatogonial stem cell self-renewal. Cell Stem Cell 12, 774–786.
Morimoto, H., Kanatsu-Shinohara, M., Shinohara, T., 2015. ROS-generating oxidase *Nox3* regulates the self-renewal of mouse spermatogonial stem cells. Biol. Reprod. 92, 147.
Nagano, M., Brinster, C.J., Orwig, K.E., Ryu, B.Y., Avarbock, M.R., Brinster, R.L., 2001. Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc. Natl. Acad. Sci. U. S. A. 98, 13090–13095.
Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y., Nakayama, K., 1997. Mice lacking $p27^{Kip1}$ display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85, 707–720.
Oatley, J.M., Avarbock, M.R., Telaranta, A.I., Fearon, D.T., Brinster, R.L., 2006. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc. Natl. Acad. Sci. U. S. A. 103, 9524–9529.
Oatley, J.M., Avarbock, M.R., Brinster, R.L., 2007. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J. Biol. Chem. 282, 25842–25851.
Oatley, J.M., Oatley, M.J., Avarbock, M.R., Tobias, J.W., Brinster, R.L., 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136, 1191–1199.
Oatley, M.J., Racicot, K.E., Oatley, J.M., 2011. Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol. Reprod. 84, 639–645.
Ogawa, T., Dobrinski, I., Avarbock, M.R., Brinster, R.L., 1998. Leuprolide, a gonadotropin-releasing hormone agonist, enhances colonization after spermatogonial transplantation into mouse testes. Tissue Cell 30, 583–588.
Ogawa, T., Dobrinski, I., Brinster, R.L., 1999. Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31, 461–472.
Ogawa, T., Dobrinski, I., Avarbock, M.R., Brinster, R.L., 2000. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat. Med. 6, 29–34.
Ogawa, T., Ohmura, M., Tamura, Y., Kita, K., Ohbo, K., Suda, T., Kubota, Y., 2004. Derivation and morphological characterization of mouse spermatogonial stem cell lines. Arch. Histol. Cytol. 67, 297–306.
Ohta, H., Wakayama, T., Nishimune, Y., 2004. Commitment of fetal male germ cells to spermatogonial stem cells during mouse embryonic development. Biol. Reprod. 70, 1286–1291.
Orwig, K.E., Shinohara, T., Avarbock, M.R., Brinster, R.L., 2002. Functional analysis of stem cells in the adult rat testis. Biol. Reprod. 66, 944–949.
Ploemacher, R.E., van der Sluijs, J.P., Voerman, J.S.A., Brons, N.H.C., 1989. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74, 2755–2763.
Resnick, J.L., Bixler, L.S., Cheng, L., Donovan, P.J., 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359, 550–551.
Ryu, B.M., Orwig, K.E., Kubota, H., Avarbock, M.R., Brinster, R.L., 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev. Biol. 274, 158–170.
Ryu, B.Y., Kubota, H., Avarbock, M.R., Brinster, R.L., 2005. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc. Natl. Acad. Sci. U. S. A. 102, 14302–14307.
Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y., Ogawa, T., 2011a. In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471, 504–507.
Sato, T., Katagiri, K., Yokonishi, T., Kubota, Y., Inoue, K., Ogonuki, N., Matoba, S., Ogura, A., Ogawa, T., 2011b. In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat. Commun. 2, 472.
Sato, T., Yokonishi, T., Komeya, M., Katagiri, K., Kubota, Y., Matoba, S., Ogonuki, N., Ogura, A., Yoshida, S., Ogawa, T., 2012. Testis tissue explantation cures spermatogenic failure in c-Kit ligand mutant mice. Proc. Natl. Acad. Sci. U. S. A. 109, 16934–16938.
Sato, T., Katagiri, K., Kojima, K., Komeya, M., Yao, M., Ogawa, T., 2015a. In vitro spermatogenesis in explanted adult mouse testis tissues. PLoS One 10, e0130171.
Sato, T., Sakuma, T., Yokonishi, T., Katagiri, K., Kamimura, S., Ogonuki, N., Ogura, A., Yamamoto, T., Ogawa, T., 2015b. Genome editing in mouse spermatogonial stem cell lines using TALEN and double-nicking CRISPR/Cas9. Stem Cell Rep. 5, 75–82.
Shinohara, T., Avarbock, M.R., Brinster, R.L., 1999. β_1- and α_6-integrin are surface markers on mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. U. S. A. 96, 5504–5509.
Shinohara, T., Orwig, K.E., Avarbock, M.R., Brinster, R.L., 2001. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc. Natl. Acad. Sci. U. S. A. 98, 6186–6191.
Shinohara, T., Kato, M., Takehashi, M., Lee, J., Chuma, S., Nakatsuji, N., Kanatsu-Shinohara, M., Hirabayashi, M., 2006. Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. Proc. Natl. Acad. Sci. U. S. A. 103, 13624–13628.
Shinohara, T., Ishii, K., Kanatsu-Shinohara, M., 2011. Unstable side population phenotype of mouse spermatogonial stem cells in vitro. J. Reprod. Dev. 57, 288–295.
Spinnler, K., Köhn, F.M., Schwarzer, U., Mayerhofer, A., 2010. Glial cell line- derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man. Hum. Reprod. 25, 2181–2187.
Stevens, L.C., 1966. Development of resistance to teratocarcinogenesis by primordial germ cells in mice. J. Natl. Cancer Inst. 37, 859–867.
Tadokoro, Y., Yomogida, K., Ohta, H., Tohda, A., Nishimune, Y., 2002. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech. Dev. 113, 29–39.
Takase, H.M., Nusse, R., 2016. Paracrine Wnt/β-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. Proc. Natl. Acad. Sci. U. S. A. 113, E1489–E1497.
Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Takehashi, M., Morimoto, H., Shinohara, T., 2011. Rac mediates mouse spermatogonial stem cell homing to germline niches by regulating transmigration through the blood-testis barrier. Cell Stem Cell 9, 463–475.
Takashima, S., Hirose, M., Ogonuki, N., Ebisuya, M., Inoue, K., Kanatsu-Shinohara, M., Tanaka, T., Nishida, E., Ogura, A., Shinohara, T., 2013. Regulation of pluripotency in male germline stem cells by Dmrt1. Genes Dev. 27, 1949–1958.
Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Morimoto, H., Inoue, K., Ogonuki, N., Jijiwa, M., Takahashi, M., Ogura, A., Shinohara, T., 2015. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep. 4, 489–502.
Takehashi, M., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Ogura, A., Shinohara, T., 2007. Adenovirus-mediated gene delivery into mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. U. S. A. 104, 2596–2601.
Takeishi, S., Nakayama, K.I., 2014. Role of Fbxw7 in the maintenance of normal stem cells and cancer-initiating cells. Br. J. Cancer 111, 1054–1059.
Tanaka, T., Kanatsu-Shinohara, M., Hirose, M., Ogura, A., Shinohara, T., 2015. Pluripotent cell derivation from male germline cells by suppression of *Dmrt1* and *Trp53*. J. Reprod. Dev. 61, 473–484.
Tanaka, T., Kanatsu-Shinohara, M., Lei, Z., Rao, C.V., Shinohara, T., 2016. The luteinizing hormone-testosterone pathway regulates mouse spermatogonial stem cell self-renewal by suppressing WNT5A expression in Sertoli cells. Stem Cell Rep. 7, 1–13.
Tomita, H., Tanaka, K., Tanaka, T., Hara, A., 2016. Aldehyde dehydrogenase 1A1 in stem cells and cancer. Oncotarget 7, 11018–11032.
Trefil, P., Micáková, A., Mucksová, J., Hejnar, J., Poplstein, M., Bakst, M.R., Kalina, J., Brillard, J.P., 2006. Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. Biol. Reprod. 75, 575–581.
Valli, H., Sukhwani, M., Dovey, S.L., Peters, K.A., Donohue, J., Castro, C.A., Chu, T., Marshall, G.R., Orwig, K.E., 2014. Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. Fertil. Steril. 102, 566–580.
van Dissel-Emiliani, F.M., de Boer-Brouwer, M., de Rooij, D.G., 1996. Effect of fibroblast growth factor-2 on Sertoli cells and gonocytes in coculture during the perinatal period. Endocrinology 137, 1885–1893.
Vendrell, M., Park, S.J., Chandran, Y., Lee, C.L., Ha, H.H., Kang, N.Y., Yun, S.W., Chang, Y.T., 2012. A fluorescent screening platform for the rapid evaluation of chemicals in cellular reprogramming. Stem Cell Res. 9, 185–191.
Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., Xie, Z., Bai, M., Yin, Q., Liang, D., et al., 2015. Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res. 25, 67–79.
Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., Jaenisch, R., 2013a. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370–1379.
Yang, Q.E., Kim, D., Kaucher, A., Oatley, M.J., Oatley, J.M., 2013b. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. J. Cell Sci. 126, 1009–1020.
Yeh, J.R., Zhang, X., Nagano, M.C., 2011. Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. J. Cell Sci. 124, 2357–2366.
Yeh, J.R., Zhang, X., Nagano, M.C., 2012. Indirect effects of Wnt3a/β-catenin signalling support mouse spermatogonial stem cells in vitro. PLoS One 7, e40002.
Yokonishi, T., Sato, T., Komeya, M., Katagiri, K., Kubota, Y., Nakabayashi, K., Hata, K., Inoue, K., Ogonuki, N., Ogura, A., et al., 2014. Offspring production with sperm grown in vitro from cryopreserved testis tissues. Nat. Commun. 5, 4320.
Zhang, F.P., Poutanen, M., Wilberts, J., Huhtaniemi, I., 2001. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol. Endocrinol. 15, 172–183.
Zhang, X., Ebata, K.T., Nagano, M., 2003. Genetic analysis of the clonal origin of regenerating spermatogenesis following transplantation. Biol. Reprod. 69, 1872–1878.
Zohni, K., Zhang, X., Tan, S.L., Chan, P., Nagano, M., 2012. CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes. Biol. Reprod. 87 (27).